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# A rapid and simple screening method for pathogenicity of *Rhizoctonia solani* and *Macrophomina phaseolina* on cotton seedlings

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**Abstract**

Cell-free15-day-old culture filtrates of 19 isolates of *Rhizoctonia solani* and 19 isolates of *Macrophomina phaseolina* differed significantly in their capacity to inhibit germination of cottonseeds after 4 days of incubation and to inhibit elongation of radicle after 12 days of incubation. Data for pathogenicity of *R. solani* and *M. phaseolina* isolates and each of seed germination and radicle length were entered into a computerized linear regressions analysis, which constructed six predictive models by using seed germination or radicle length, singly or in combination, as physiological predictors. It was found that the radicle length model (r =-0.936, p=0.000) and the seed germination model (r=-0.851, p=0.000) were the best models for predicting pathogenicity of *R. solani* and *M. phaseolina* isolates, respectively. The results of the present study suggest that cell-free culture filtrate assay reflects the relative pathogenicity of the various isolates of *R. solani* and *M. phaseolina* and may provide a practical complimentary method to greenhouse tests.

**Key words** – cotton – culture filtrates – *Macrophomina phaseolina* – pathogenicity – *Rhizoctonia solani*

## Introduction

*Rhizoctonia solani* Kühn (sexual stage: *Thanatephorus cucumeris* (Frank) Donk) is one of the more primitive basidiomycetes. *R. solani* exists in its vegetative form in nearly all agricultural soils. In this non-spore producing phase, the fungus lives saprophytically on dead plant remains, but it can become vigorously parasitic when roots or other parts of a susceptible host penetrate the infested zone (Watkins 1981). Current classification of *R. solani* is based largely on grouping of isolates into anastomosis groups ($AG_s$). Anastomosis, or the fusion of hyphae between individuals, may result in the sharing of genetic material without sexual reproduction, but it also serves to isolate individuals from other members of the same species that do not share the alleles for somatic compatibility (Agrios 2005).

Pathogenicity of *R. solani* on Egyptian cotton (*Gossypium barbadense* L.) is well documented in the literature. For example, Mostafa-Mahmoud et al. (1995) and Asran-Amal et al.

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(2005) reported that *R. solani* was a major cause of cotton damping-off throughout much of the cotton-growing areas in Egypt. El-Akkad (1997) evaluated the pathogenicity of 39 Egyptian isolates of *R. solani* AG-4 and one isolate AG-2-2 under greenhouse conditions on cotton cultivar Giza 75. Most of the virulent isolates exhibited pre-emergence damping-off. She also found that 20 isolates of *R. solani* recovered from naturally infected cotton seedlings belonged to AG-5, and two belonged to unidentified AG. Seventeen isolates of *R. solani* AG-4 and an isolate of binucleate *Rhizoctonia* were tested for pathogenicity on seedlings of cotton cultivar Giza 83. All isolates were so virulent that they caused 100% mortality in the pre-emergence stage (El-Samawaty 1999). Eighteen isolates of *R. solani* AG-4 were tested for pathogenicity on seedlings of cotton cultivars Giza 86 and Giza 83, ten isolates were so virulent that they caused more than 95% mortality in pre-emergence stage (Asran-Amal 2001). Fifty-two isolates of *R. solani* were isolated from cotton seedlings infected with post-emergence damping-off disease. Of these, 13–46% belonged to AG-2-2, 25% belonged to AG-4-AGI, and 61.54% were AG-4-4-HGII (El-Samawaty et al. 2008). Pathogenicity test of 51 isolates of *R. solani* on cotton cultivar Giza 86, under greenhouse conditions, showed that 19 isolates significantly induced pre- and post-emergence damping-off, while they significantly decreased survival, plant height, and dry weight. The pathogenic isolates of AG-2-2 represented 19.61% of the total isolates as well as the highest percentage (52.63%) of the pathogenic isolates (Kasem 2009). Eighty-two isolates of *R. solani* were recovered from roots of naturally infected seedlings of the Egyptian cottons, three (3.7%) of the isolates were identified as *R. solani* AG-7, while the remaining isolates belonged to the AG-2-1, AG-4, and AG-5. Pathogenicity tests under greenhouse conditions revealed that AG-7 caused the common symptoms of damping-off, which included seed rot, lesions on the hypocotyle, and root rot (Abd-Elsalam et al. 2010).

*Macrophomina phaseolina* (Tassi) Goid., the casual agent of charcoal rot (ashy stem) on cotton, is a seed-borne and a soil-borne pathogen with a wide distribution and a wide host range (Dhingra & Sinclare 1978). When *M. phaseolina* invades roots or stems of cotton, colonization of internal tissues proceeds rapidly and the plant dies. Examination of affected parts reveals a dry rot, with many tiny black sclerotia distributed throughout the wood and softer tissues (Watkins 1981). A negative correlation ($r = -0.85$, $p<0.01$) was found between disease incidence and yield (Turini et al. 2000).

*M. phaseolina* has a widespread distribution in Egyptian soil, and it is easily and frequently isolated from cotton roots particularly during the late period of the growing seasons. Thus, when Aly et al. (1996) conducted a survey encompassing 88 samples of infected cotton roots from 12 governorates, *M. phaseolina* was isolated from 37.5% of the samples examined. Although initial infections of cotton by *M. phaseolina* occur at the seedlings stage, they usually remain latent until the cotton plant approaches maturity (Dhingra & Sinclare 1978). *M. phaseolina* appears to affect some cotton cultivars less severely than others, suggesting the existence of some level of resistance to *M. phaseolina* (Watkins 1981, Lee et al. 1986, Monga & Rai 1996, 2000, Turini et al. 2001). Many plant pathogenic fungi produce host-specific phytotoxins that are primary determinants in pathogenesis and induce typical disease symptoms in the absence of the pathogen (Song et al. 1994). Toxic culture filtrates and purified toxins have been widely used for *in vitro* selection and regeneration of disease resistant plants (Song et al. 1994). On the contrary, to the best of our knowledge, only two attempts have been made to assess the range of pathogenicity within a plant pathogenic fungus by using toxic culture filtrates (Madhosingh 1995, Xu et al. 2004).

Differentiation among isolates of plant pathogenic fungi is important for improving our understanding of the ecology of these isolates and the epidemiology of the diseases caused by pathogenic fungi. The conventional method of differentiation among pathogen isolates is the observation of the differences in virulence when the isolates interact with a set of host genotypes under greenhouse conditions (Aly et al. 2007, Kasem 2009). However, this method is often laborious, time-consuming, produces low symptom expression, requires considerable greenhouse space, and carries a high chance of cross contamination during inoculation or soil infestation

(Horowitz et al. 2004). Furthermore, the method may be influenced by variability inherent in the experimental system (Aly 1988, Bhatti & Kraft 1992).

Therefore, another reliable method, either alternative or complementary to that based on the differential interaction between pathogen isolates and host genotypes, is required for differentiation among *R. solani* or *M. phaseolina* isolates from cotton. The use of toxic culture filtrates could be a good candidate to achieve this goal. This method is a rapid, dependable screening procedure that minimizes the previously mentioned difficulties and facilitates a large scale screening of *R. solani* or *M. phaseolina* isolates (Kuti et al. 1997). However one should keep in mind that a necessary condition for a ready use of toxic culture filtrates as a tool for screening pathogen isolates is the presence of a correlation between *in vitro* response to the pathogen culture filtrates and *in vivo* behavior of the pathogen (Buiatti et al. 1985).

Therefore, the first objective of the present study was to investigate the phytotoxic effects of culture filtrates of *R. solani* and *M. phaseolina* isolates from cotton roots on cotton seeds. The second objective was to assess the correlation between the *in vitro* toxicity of the isolates and their pathogenicity under greenhouse conditions.

## Materials & Methods

### Fungal isolates and production of inoculum

Isolates of *R. solani* and *M. phaseolina* (Tables 1, 2) used in the present study were obtained from the fungal collection of the Cotton and the Fiber Crops Diseases Research Section, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. All isolates were originally isolated from cotton roots. A substrate for growth of isolates was prepared in 500 ml glass bottles, each contained 50 g of sorghum grains and 40 ml of tap water. Contents of each bottle were autoclaved for 30 minutes. Isolate inoculums, taken from one-week-old culture in potato dextrose agar (PDA) medium, was aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks.

### Pathogenicity tests of *R. solani* and *M. phaseolina* isolates

Pathogenicity tests were carried out by using autoclaved clay loam soil. Batches of soil were separately infested with inoculum of each isolate at rates of 1 and 50 g/kg of soil for *R. solani* and *M. phaseolina*, respectively. Infested soil was dispensed in 10 cm diameter clay pots and these were planted with 10 seeds per pot (cultivar Giza 89). In the control treatments, sterilized sorghum grains were mixed thoroughly with soil at the previously mentioned rates. Pots were randomly distributed on a greenhouse bench. The prevailing temperature during pathogenicity test ranged from 21.5ºC±3.5 to 31.0±3.0ºC. Pathogenicity (percentage of dead seedlings) was recorded 45 days after planting.

### Toxicity of culture filtrates of *R. solani* and *M. phaseolina* isolates on germination of cottonseeds and elongation of radicle

Substrate for growth of each of the tested isolates was prepared in 100 ml conical flasks, each flask contained 50 ml of sterilized Czapeck's liquid medium. Isolate inoculum, taken from one-week-old culture on PDA, was aseptically introduced into the flask and allowed to grow in the medium for 15 days. There were five flasks for each isolate. The inoculated flasks were randomly distributed on a laboratory bench under temperature regime from 16.5±1.5 Cº to 29±1 Cº. Culture filtrates of the isolate were obtained through Whatman's No.1 filter paper. Microscopic examination of culture filtrates did not show the presence of any mycelium. Five sterilized cotton seeds of cultivar Giza 89 were plated on a sterilized cotton layer per plate. Twenty ml of filtrate of each isolate were added to each plate. In the control treatments, sterilized distilled water was added to plates. There were five plates for each isolate. The plates were randomly distributed on a laboratory bench under the previously mentioned temperature regime. After incubation for 4 days,

percentage of germinating seeds was recorded. Length of radicle was recorded 12 days from incubation. All tests and experiments were repeated once.

**Statistical analysis**

The experimental design of greenhouse pathogenicity tests and the laboratory experiments was randomized complete block with five replicates (blocks). Analysis of variance (ANOVA) of the data was performed with MSTAT-C. Least significant difference (LSD) was used to compare isolate means. Linear regression analysis was used to evaluate the relationship between seed germination and length of radicle (independent variables) and pathogenicity (dependent variable). Regression analysis was performed with a computerized program (SPSS version 13).

**Results**

In culture filtrate bioassay, all crude culture filtrates of *R. solani* isolates significantly reduced seed germination and radicle elongation (Table 1). However, the isolates showed a considerable level of variation regarding the toxicity of their culture filtrates. Thus, isolate R18 was the most toxic to seed germination as it reduced it by 75.68%, while isolate R10 displayed the least toxicity as it reduced seed germination by 43.24%. As to radicle length, isolate R18 was also the most toxic reducing it by 83.68%, while isolate R1 was the least toxic reducing it by 51.95%.

Data for pathogenicity of *R. solani* or *M. phaseolina* isolates and each of seed germination and radicle length were entered into a computerized linear regression analysis, which constructed six predictive models by using seed germination or radicle length, singly or in combination, as physiological predictors (Table 3, Figs 1, 2). Pathogenicity of *R. solani* was negatively correlated with each of seed germination (r=-0.882, p=0.000) and radicle length (r=-0.936, p=0.000). However, pathogenicity of *M. phaseolina* was negatively correlated with seed germination (r=-0.851, p=0.000) and was not significantly correlated with radicle length (r=0.256, p=0.276). The multiple regression models, which involved the two predictors, showed the highest $R^2$ values.

**Discussion**

Cell-free 15-day-old culture filtrates of 19 isolates of *R. solani* and 19 isolates of *M. phaseolina* differed significantly in their capacity to inhibit germination of cottonseed after four days of incubation and to inhibit elongation of radicle after 12 days of incubation. These findings are in agreement with previous studies, which reported inhibitory effects of culture filtrates of *R. solani* (Gurha & Vishwadhar 2003, Deshpande et al. 2006, Anjana & Kumar 2007, Yadav 2012) and *M. phaseolina* (Jhutha et al. 1997, Kuti et al. 1997) isolates on seed viability of different crops. Isolates of *R. solani* and *M. phaseolina* are known to produce biologically active toxins in their culture filtrates. Phenyl acetic acid and its hydroxy derivatives are some of the toxins produced by *R. solani* (Deshpande et al. 2006). Phsaeolinaone is the toxin produced by *M. phasoelina* (Kuti et al. 1997). Therefore, it seems reasonable to suggest that the observed inhibition in seed germination and radicle elongation was elicited by the toxins in culture filtrates. All the culture filtrates from the different isolates of *R. solani* and *M. phaseolina* were tested by using the seeds of cultivar Giza 89. Hence, it appears that the observed differences in toxicity capacity of filtrates from the isolates were due, at least in part, to the inherent differences among the isolates in producing toxins.

The composition of the growth medium is an important factor in inducing effective toxic activity by culture filtrates of pathogens (Madhosingh 1995, Duarte & Archer 2003). In this study, Czapeck's liquid medium produced considerable toxic effects when it was used as a growth medium for *R. solani* and *M. phaseolina* isolates, which may indicate that this medium would be a good candidate for future culture filtrate studies.

A computerized linear regression analysis generated six models for predicting pathogenicity of *R. solani* and *M. phaseolina* isolates. In these models, seed germination and radicle length were used, singly or in combination, as physiological predicators. At this point, the question that may arise as to which of these models are the best models for predicting pathogenicity of the isolates. In the case of *R. solani*, when seed germination and radicle length were used as predictors, the $R^2$

**Table 1** Effect of filtrates of *Rhizoctonia solani* isolates on seed germination, length of radicle of cotton cultivar Giza 89, and pathogenicity of the isolates on this cultivar under greenhouse conditions.

| Isolates | Anastomosis group (AG) | Geographic origin | | Seed germination (%) after 4days | Radicle length (mm) after 12 days | Pathogenicity (%) |
|---|---|---|---|---|---|---|
| | | Governorate | Region | | | |
| R1 | 4 | Sharqiya | East Delta | 50.00 | 5.30 | 64.00 |
| R2 | 2-2 | Beheira | West Delta | 42.50 | 4.00 | 60.00 |
| R3 | 2-2 | Daqahliya | East Delta | 40.00 | 4.25 | 61.00 |
| R4 | 4 | Gharbiya | Middle Delta | 45.00 | 4.30 | 64.00 |
| R5 | 4 | Daqahliya | East Delta | 50.00 | 4.80 | 62.00 |
| R6 | 2-2 | Gharbiya | Middle Delta | 35.00 | 3.00 | 78.00 |
| R7 | 2-2 | Kafr El-Sheikh | North Delta | 37.00 | 3.20 | 72.00 |
| R8 | 4 | Kafr El-Sheikh | North Delta | 30.00 | 2.50 | 88.00 |
| R9 | 2-2 | Sharqiya | East Delta | 45.00 | 4.00 | 64.00 |
| R10 | 2-2 | Beheira | West Delta | 52.50 | 5.00 | 58.00 |
| R11 | 4 | Sharqiya | East Delta | 40.00 | 3.85 | 61.00 |
| R12 | 4 | Minufiya | South Delta | 30.00 | 2.50 | 90.00 |
| R13 | 4 | Minufiya | South Delta | 37.50 | 2.30 | 88.00 |
| R14 | 2-2 | Assuit | Upper Egypt | 30.00 | 2.00 | 89.00 |
| R15 | 4 | Sohag | Upper Egypt | 35.00 | 2.30 | 90.00 |
| R16 | 4 | Assuit | Upper Egypt | 22.50 | 2.30 | 92.00 |
| R17 | 2-2 | Minya | Middle Egypt | 25.00 | 2.00 | 88.00 |
| R18 | 4 | Sohag | Upper Egypt | 25.00 | 1.80 | 91.50 |
| R19 | 4 | Damietta | East Delta | 45.00 | 4.00 | 63.00 |
| Control[a] | | | | 92.50 | 11.03 | 5.00 |
| **LSD($p \leq 0.05$)** | | | | 10.25 | 0.47 | 5.62 |

[a]In the case of seed germination and radicle length, control denotes sterilized distilled water, while it denotes autoclaved soil in the case of pathogenicity. LSD ($p \leq 0.05$)

values of the generated models were 0.778 and 0.875, respectively, making radicle length better suited for use in screening pathogenicity of the isolates. The increase in the $R^2$ values of the multiple model ($R^2$ values=0.877) was too small to justify the use of this model in prediction. In the case of *M. phaseolina*, seed germination was the only suitable single variable for prediction ($R^2$=0.724). In addition to the high $R^2$ value, seed germination is an easily acquired variable. On the other hand, it is inadvisable to use the multiple models as its application took 12 days compared with the 4 days required for the application of the seed germination model. Therefore, it was concluded that radicle length model and spore germination model were the best models for predicting pathogenicity of *R. solani* and *M. phaseolina* isolates, respectively. However, one should keep in mind that the use of these models is a complimentary and not an alternative method to the greenhouse tests. Evidently, the use of these models would considerably reduce the number of isolates, which are tested under greenhouse conditions. The significant negative correlations of these models suggest that toxins were also the operative mechanisms, which elicited damping-off of seedlings in the greenhouse tests.

Since the present study is preliminary in nature, it remains to determine the environmental conditions required for the optimization of culture filtrate bioassay procedures.

**Table 2** Effect of filtrates of *Macrophmina phaseolina* isolates on seed germination, length of radicle of cotton cultivar Giza 89, and pathogenicity of the isolates on this cultivar under greenhouse conditions.

| Isolates | Geographic origin | | Seed germination (%) after 4days | Radicle length (mm) after 12days | Pathogenicity (%) |
|---|---|---|---|---|---|
| | Governorate | Region | | | |
| M1 | Kafr El-Sheikh | North Delta | 47.50 | 7.63 | 40.00 |
| M2 | Daqahliya | East Delta | 35.00 | 12.25 | 75.00 |
| M3 | Daqahliya | East Delta | 40.00 | 6.45 | 77.50 |
| M4 | Sharqiya | East Delta | 30.00 | 7.75 | 85.00 |
| M5 | Kafr El-Sheikh | North Delta | 35.00 | 14.65 | 85.00 |
| M6 | Sharqiya | East Delta | 20.00 | 10.08 | 87.50 |
| M7 | Minufiya | South Delta | 22.50 | 5.20 | 85.00 |
| M8 | Demietta | East Delta | 45.00 | 7.93 | 65.00 |
| M9 | Qualyubiya | South Delta | 27.50 | 3.33 | 90.00 |
| M10 | Minufiya | South Delta | 20.00 | 6.75 | 90.00 |
| M11 | Qualyubiya | South Delta | 27.50 | 9.10 | 82.50 |
| M12 | Beheira | West Delta | 45.00 | 4.90 | 87.50 |
| M13 | Beheira | West Delta | 20.00 | 8.88 | 90.00 |
| M14 | Gharbiya | Middle Delta | 17.50 | 7.75 | 95.00 |
| M15 | Assuit | Upper Egypt | 35.00 | 10.55 | 85.00 |
| M16 | Assuit | Upper Egypt | 22.50 | 16.43 | 90.00 |
| M17 | Gharbiya | Middle Delta | 35.00 | 7.55 | 77.50 |
| M18 | Sohag | Upper Egypt | 20.00 | 14.10 | 92.50 |
| M19 | Sohag | Upper Egypt | 40.00 | 10.95 | 75.00 |
| Control | | | 87.50 | 20.00 | 5.00 |
| LSD($p \leq 0.05$) | | radicle | 12.34 | 2.24 | 9.13 |

[a]In the case of seed germination and radicle length, control denotes sterilized distilled water, while it denotes autoclaved soil in the case of pathogenicity.

**Table 3** Linear regression models that describe that relationship between *in vitro* seed germination (X1) or *in vitro* radicle length (X2) of cotton cultivar Giza 89 and *in vivo* pathogenicity (Y) of *R. solani or M. phaseolina* isolates on this cultivar under greenhouse conditions.

| Pathogen | Model no. | Independent variable (predictor) | Linear regression model | r[a] | $R^{2b}$ | F.value | P> F |
|---|---|---|---|---|---|---|---|
| *R. solani* | 1 | Seed germination(X1) | Y=125.140-1.3285X1 | -0.882 | 0.778 | 59.558 | 0.000 |
| | 2 | Radicle length (X2) | Y=112.618- 11.281X2 | -0.936 | 0.875 | 119.41 | 0.000 |
| | 3 | X1 + X2 | Y=114.593-1.521X1-10.152X2 | -0.936 | 0.877 | 56.935 | 0.000 |
| *M. phaseolina* | 4 | Seed germination(X1) | Y=113.944-1.0694X1 | -.851 | 0.724 | 47.287 | 0.000 |
| | 5 | Radicle length (X2) | Y=71.809+0.940X2 | 0.256 | 0.065 | 1.260 | 0.276 |
| | 6 | X1 + X2 | Y=111.937-10.546X1+0.174X2 | -0.852 | 0.726 | 22.567 | 0.000 |

[a]Linear correlation coefficient.
[b]Coefficient determination.

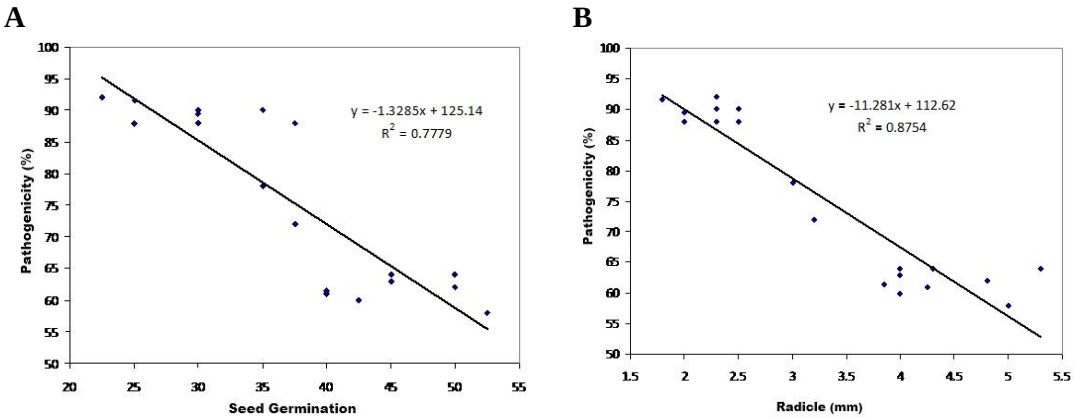


**Fig 1** – Relationship between seed germination (A) or radicle length (B) and pathogenicity (%) for *R. solani*

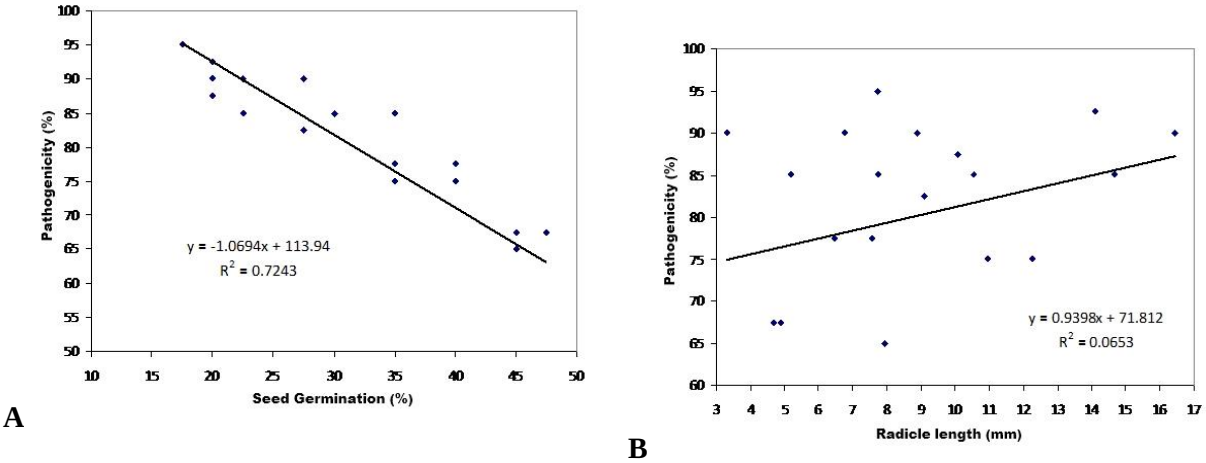


**Fig 2** – Relationship between seed germination (A) or radicle length (B) and pathogenicity (%) for *M. phaseolina*

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